Isolation and Characterization of Ultraviolet Light-Sensitive Mutants of the Blue-Green Alga *Anacystis nidulans*

YUKIO ASATO[1]

Ames Research Center, NASA, Moffett Field, California 94035

Three independently isolated ultraviolet light-sensitive (uvs) mutants of *Anacystis nidulans* were characterized. Strain uvs-1 was most sensitive to UV in the absence of photoreactivation. Pretreatment with caffeine suppressed the dark-survival curve of strain uvs-1, indicating the presence of excision enzymes involved in dark repair. Under "black" and "white" illumination, strain uvs-1 displays photoreactivation properties nearly comparable to wild-type culture. Mutants uvs-35 and uvs-88 appeared to have partial photorecovery capacities. Upon pretreatment with chloramphenicol, photoreactivation properties of strains uvs-1 and uvs-88 were not evident although the partial photoreactivation characteristics of strain uvs-35 remained the same. Data indicate that strains uvs-1, uvs-35, and uvs-88 are probably genetically distinct UV-sensitive mutants.

The blue-green algae display extremely efficient photoreactivation. For example, at an ultraviolet light (UV) dose which gives approximately 0.001% survival, total photoreactivation of all irradiated cells could be obtained. Van Baalen (18) suggested that UV primarily damages the photosynthetic apparatus and photorecovery was due to the photorepair of the photosynthetic system. Nevertheless, the presence of a photoreactivating enzyme in blue-green algae was demonstrated in vitro by Werbin and Rupert (19), who found that cell extracts of *Plectonema boryanum* photoreactivated UV-damaged transforming deoxyribonucleic acid (DNA) of *Hemophilus influenzae*. Recently, Saito and Werbin (11) purified the photoreactivation enzyme from *Anacystis nidulans* no. 625 which catalyzes the photorepair of pyrimidine dimers maximally at 435.8 nm. In addition, photoreactivation of UV-irradiated algal virus LPP-1 (20) offers direct support that photoenzymatic repair of UV-damaged DNA is a major mode of repair in vivo. Possibilities exist that in blue-green algae there are two photorecovery processes. One route could be the photoreactivation of pyrimidine dimers and the other related in some manner to photorecovery of photosynthetic apparatus.

UV-sensitive mutants and mutants of impaired photorecovery systems should provide a means for analyzing the repair of UV irradiation damage by the blue-green algae. This initial study will describe the isolation and characterization of UV-sensitive and partial photoreactivation mutants of *Anacystis*. Attempts will also be made to characterize photoreactivation of *Anacystis* and to delineate the possible modes of partial photoreactivation displayed in particular by two mutants.

MATERIALS AND METHODS

Organism. *A. nidulans* no. 625, a unicellular, obligate photoautotroph, was obtained from the algal culture collection of Indiana University (15). The culture of *Anacystis* has been purified and maintained as a pure culture.

Media and growth conditions. Dm minimal broth and agar (17) or HEPES (*N*-2-hydroxyethylpiperizine-*N'*-2'-ethanesulfonic acid; Calbiochem)-buffered Dm medium were used. Concentration of HEPES, growth conditions, and viable cell assay methods were previously described (2).

Isolation of UV-sensitive mutants. Exponential cultures of *Anacystis* were treated with a final concentration of 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG; Aldrich Chemical Co.) per ml of water for 8 min. The mutagen was removed by centrifugation at 9,000 rev/min for 5 min. The supernatant fluid was discarded and fresh Dm-HEPES broth was added to the pellet. The mutagenized cul-

[1] Present address: Department of Biology, Southeastern Massachusetts University, No. Dartmouth, Mass. 02747.

ture was incubated under fluorescent light for 3 to 4 days to allow for segregation. An incubated culture was diluted, and 0.1 ml was spread on agar to obtain approximately 300 colonies per plate. Agar plates were incubated for 2 to 3 days or until the appearance of colonies barely visible to the unaided eye. Colonies were then directly exposed to a UV lamp (G.E., 15-w germicidal) for 1 min at a distance of 20 cm. Irradiated plates were exposed to unfiltered "black" light (Ultra-Violet Products, Inc., San Gabriel, Calif., BLB 30 wavelength distribution, 300–400 nm; maximum emittance, 366 nm; emission lines at 435.8 and 546.1 nm, 227% and 114%, respectively, as compared to emission line 366.0 nm which is taken as 100%) at a distance of approximately 10 to 15 cm for 3 hr. This photoreactivation period was stopped by placing the plates in the dark for 24 hr. To permit growth, the plates were subsequently incubated under fluorescent lights. In approximately 7 days, suspected mutants appearing as small colonies were picked and restreaked. Each isolate was screened for its ability to repair UV damage.

UV-irradiated dark-survival curves. An exponential culture in Dm broth (absorbance 0.041 at 254 nm) or Dm-HEPES broth (absorbance 0.2 at 254 nm) was irradiated in a darkened room. Three milliliters of culture was placed in a small petri dish (55-mm diameter) and irradiated with the germicidal lamp at a distance of 20 cm for increments of about 5 sec. Samples of 0.01 ml were put in test tubes containing phosphate-buffered diluent. Tubes were completely covered with aluminum foil and left at room temperature for approximately 24 hr. Samples were then diluted, plated, and incubated under white fluorescent light. Enumeration of colonies was made with the aid of a dissecting microscope after 2 to 4 days of incubation.

UV-irradiated survival curves in the light. Cultures of *Anacystis* were UV-irradiated as above. Samples in petri dishes were exposed to white light (G.E., deluxe cool white, 40-w) or black light for 2 to 3 hr. Samples of 0.01 ml were placed in test tubes containing phosphate buffer. Tubes were wrapped with aluminum foil and kept at room temperature for 24 hr. Plating and incubation were done in the usual manner.

Effect of caffeine on UV-irradiated culture. Two per cent caffeine (Calbiochem) was prepared with distilled water as diluent. Exponential-phase cultures were pretreated for 5 hr with 0.2% caffeine (final concentration) and centrifuged to remove the caffeine, and the pellets obtained were resuspended in Dm broth. UV irradiation and subsequent postirradiation treatment were performed in a manner identical to those for obtaining the dark-survival curve.

Kinetics of photorecovery with black and white fluorescent lights. Exponential cultures were exposed to UV light to obtain approximately 1% survivors. UV-treated cultures were exposed to black light at increments of 10 min up to 120 min at room temperature. Irradiated samples were subjected to a 24-hr dark period and plated in the usual manner. Similar studies were performed substituting white light

(G.E., deluxe cool white, T40) for black light illumination. To characterize further the photorecovery process, similar experiments were made with cultures pretreated with 1.0 μg of chloramphenicol (Calbiochem) per ml (final concentration) for 5 hr. Culture was diluted approximately three- to fivefold with Dm medium. Chloramphenicol was either removed by centrifugation or added to maintain the concentration at 1.0 μg/ml. Irradiation and subsequent photoreactivation experiment was performed as above.

Effect of red light on UV-irradiated culture. UV irradiation was performed in the usual manner. Samples were exposed to fluorescent light (G.E., deluxe cool white) filtered through a filter (Corning) transmitting essentially red light. The 50% transmittance value for filter CS no. 2-73 is at wavelength 590 nm. The distance of the culture in an open petri dish (i) to the fluorescent light was about 20 cm, (ii) to the filter was about 8 cm. The radiant power of the deluxe cool white fluorescent lamp peaks at about 615 nm, and the spectral emittance ranges from 350 to 750 nm. At the peak height, the rated power of the lamp is about 155 μwatts per 100 nm per lumen. The glass filter utilized (Corning no. 2-73) should transmit sufficient radiant power to allow photosynthesis and growth. To obtain exponential growth, cultures of *Anacystis* were exposed under the same lighting system, although the distance was about 55 cm.

RESULTS

UV-sensitive mutants. Of the 6,415 colonies scanned, 119 suspected small colonies were picked and restreaked. Three isolates were minute colony-forming mutants and therefore were discarded. The remaining 116 colonies formed large colonies upon restreaking and were screened for the ability to photorepair UV damage. Each isolate was grown in Dm broth and UV-irradiated to obtain approximately 1% survivors. Viable assays for colony-forming units were performed on UV-irradiated control. Isolates which showed 100% photorecovery were discarded. However, 16 isolates showed some degree of impaired photorecovery processes; of these, three independently isolated stable mutants, uvs-1, uvs-35, and uvs-88, were selected for further analysis. Although an extensive search was not conducted, completely photoreactivation-less mutants were not found.

UV-irradiated dark-survival curves. *Anacystis*, being an obligate photoautotroph, requires light for growth. However, in the postirradiation period, it was found to be convenient to impose a 24-hr dark period before incubation in the light. It was necessary, therefore, to learn more about the response of an irradiated culture to a 24-hr dark period. For this purpose

Anacystis was irradiated and samples were kept in the dark for periods of 1 to 19 hr, exposed to white light for 4 hr, and then plated for growth. The results of this experiment, given in Fig. 1, show a survival curve with a slight shoulder, and an exponential slope followed by a plateau starting at about the 11th hour. Apparently, there is a lag during which the lethal processes of UV damage occur very slowly in the dark until all UV damage (minus dark repair) is expressed. The lethal processes can be immediately stopped, and the remaining damaged sites can be photorepaired if samples are exposed to light at any time before the end of the lag.

Light at wavelengths promoting photosynthesis may have a pronounced effect on the survival values of UV-irradiated cultures. To test this possibility, UV-irradiated samples were exposed to light filtered through a glass filter (Corning, CS 2-73) for 10 to 60 min, kept in the dark for 24 hr, and subsequently plated for growth in the usual manner. Figure 2 shows that red light (580 nm and above) does not induce photorecovery during an illumination period of 1 to 2 hr. In fact, light at wavelengths that promote photosynthesis appears to enhance UV damage.

The dark-survival curves (Fig. 3) demonstrate the typical responses of the *Anacystis* strains to increasing doses of UV light in the absence of photoreactive processes. In general, all curves display a lag, followed by an exponential decay. Since uvs-1 is the most sensitive, an attempt was made to determine whether this mutant lacks dark repair. It was
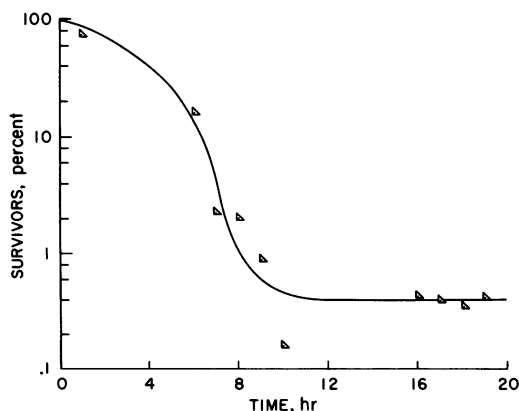

FIG. 2. *Effect of transmitted red light on UV-irradiated Anacystis culture. Irradiated wild-type cultures of Anacystis were exposed to (curve 2) cool white light and to (curve 3) cool white light filtered through a glass filter (Corning CS no. 2-73; 50% transmittance at 580 nm). After exposure to the light sources as described, samples were kept in the dark for 24 hr. Subsequently, the samples were plated under white light. As a control, unirradiated wild-type culture (curve 1) was exposed to red light and was processed as in curves 2 and 3.*
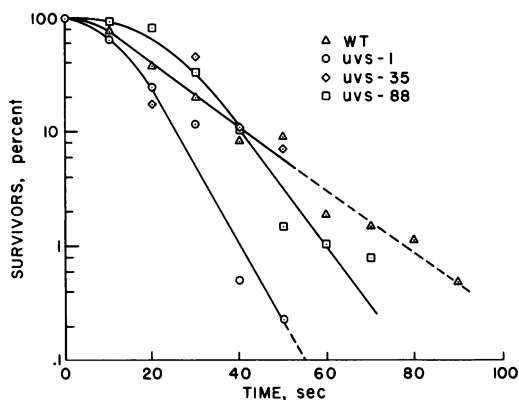
FIG. 3. *Dark-survival curve. Samples of Anacystis strains were irradiated as indicated in the text, kept in the dark for 24 hr, and subsequently plated.*

assumed that the primary target of UV irradiation is the DNA.

To an exponential culture of strain uvs-1, caffeine (final concentration 0.2%) was added. This concentration of caffeine inhibits cell growth. The mode of inhibition of caffeine in *Anacystis* was not investigated. After 5 hr the caffeine was removed by centrifugation, and fresh Dm broth was added to the pellet. Because *Anacystis* does not incorporate labeled amino acid or labeled inorganic phosphates and cell division is depressed in the dark (2), further addition of caffeine was thought not to

FIG. 1. *Survival curve of UV-irradiated Anacystis in the dark. An exponential culture of Anacystis was irradiated and kept in the dark for 0 to 19 hr at room temperature. Samples were exposed to white light for 2 hr and then plated on Dm agar.*
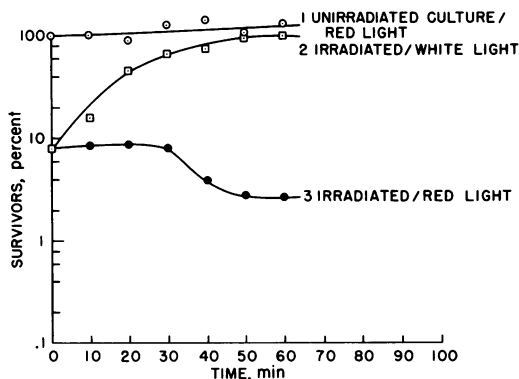
be necessary. UV light was applied in the dark to caffeine-pretreated strain uvs-1 and also to an untreated culture. Figure 4 indicates that strain uvs-1 and wild type respond to caffeine by increased sensitivity to radiation treatment. Comparison of the dark-survival curves of strain uvs-1 in Fig. 3 and Fig. 4 is not encouraged since dark-survival curves differ somewhat from one experiment to the other. However, significant differences in the dark-survival curves are repeatedly seen when one analyzes a given set of results from a given experiment generated by wild-type and mutant strains. In fact, all experiments reported in this communication are designed to show the relative response of wild-type and mutant strains to UV irradiation alone or to irradiation plus certain chemical inhibitors. In this manner, all data presented are reproducible.

Effect of black and white light on UV-irradiated culture. Photoreactivation capabilities of the mutants uvs-1, uvs-35, and uvs-88 have been studied to learn whether these mutants may have common defects. First, mutants were given increasing doses of UV light followed by exposure to 3 hr of black light, held in the dark for 24 hr, and subsequently plated for growth. As shown in Fig. 5, strain uvs-1 tolerates the least amount of UV irradiation, followed by strains uvs-88 and uvs-35 in that order. Second, curves of the rates of photoreactivation under black light illumination display differences in the photoreactivation capacities of each mutant (Fig. 6). The calculated percentages of photoreactivation (% PR
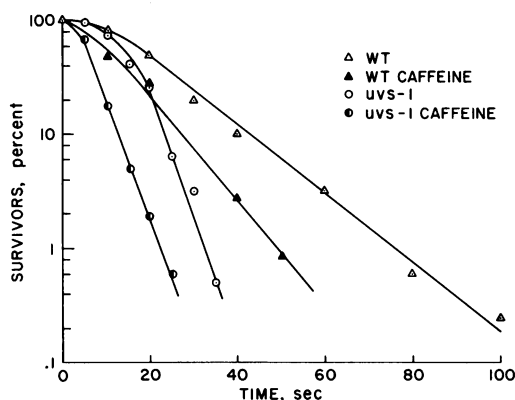

FIG. 5. *Survival curves of UV-irradiated Anacystis mutants exposed to black light. Wild-type and mutant cultures were exposed to UV irradiation as indicated and illuminated with black light for 2 hr. Samples were plated and incubated for viable cell count assays.*
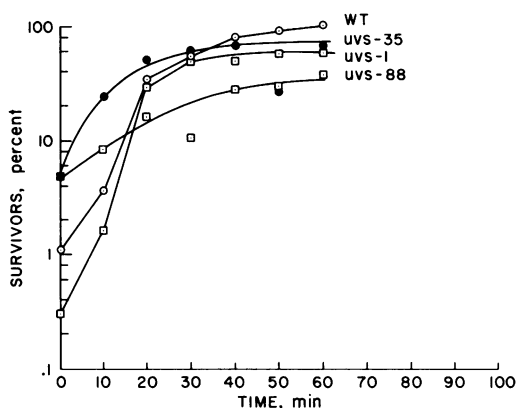
FIG. 6. *Rates of photoreactivation under black light illumination. Cultures were irradiated to obtain approximately 1% survival values. Samples were exposed to black light for 0 to 60 min at room temperature, kept in the dark for 24 hr, and subsequently plated for growth. UV doses were 40, 60, 80, and 100 sec for strains uvs-1, uvs-88, uvs-35, and wild type, respectively.*

= number of irradiated survivors after PR minus number of irradiated survivors in dark, divided by number of unirradiated cells minus number of irradiated survivors in dark, ×100) were: wild type = 100%, uvs-1 = 60%, uvs-35 = 70%, and uvs-88 = 14%. Note, however, that the dosages selected and applied to strains uvs-1, uvs-88, uvs-35, and the wild type were 40, 60, 80, and 100 sec, respectively, to obtain approximately 0.1 to 1.0% survival values. Although strain uvs-1 tolerates the least amount of UV, the % PR (60%) with black light was relatively high and the photoreactivation capacity was comparable to a wild-type culture.

FIG. 4. *Dark-survival curves of caffeine-treated cultures of wild type and strain uvs-1. Both wild-type and uvs-1 cultures were treated with 0.2% caffeine (final concentration) for 5 hr. Samples were centrifuged to remove caffeine and resuspended in Dm broth. UV irradiation was performed in the dark as in Fig. 3.*
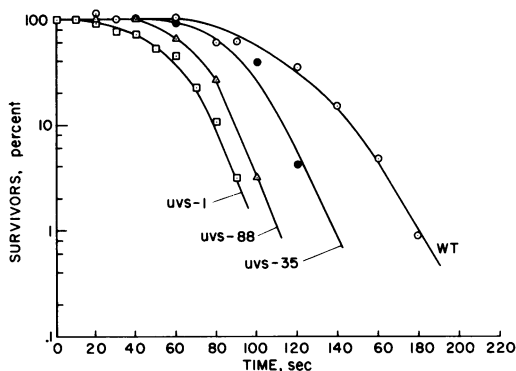
In this respect, strain uvs-1 appears to possess near normal photoreactivation capacity.

Results of photoreactivation under white light illumination (Fig. 7) were comparable to black light photoreactivation (see Fig. 6). However, in chloramphenicol-pretreated cells, mutants uvs-1 and uvs-88 displayed total inhibition in respect to white light photoreactivation whether chloramphenicol was removed (Fig. 8) or was present (experiments not shown) during the photoreactivation period investigated. The wild-type strain and strain uvs-35 showed no obvious differences between chloramphenicol-treated and untreated cultures. Although the effect of chloramphenicol was not fully investigated, the chloramphenicol experiment serves to characterize the mutants.

DISCUSSION

The lethal processes of UV-irradiated *Anacystis* occur very slowly in the dark. One would expect this slow death rate in the dark because *Anacystis* is an obligate photoautotroph. In *Escherichia coli*, conditions which induce growth delay promote greater survival of UV-irradiated cells. For example, increased survival response to growth delay was found in (i) retention of irradiated cells in buffer [liquid holding recovery (9)] and (ii) amino acid deprivation or treatment with chloramphenicol prior to irradiation of cells (6). Because DNA synthesis is inhibited in experiments restricting growth, Hanawalt (6) suggested that DNA repair prior to genome replication leads to a corresponding increase in cell survival.
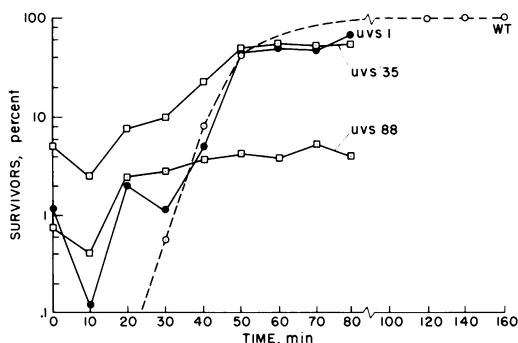
Residual metabolic activity in *Anacystis*

FIG. 7. *Rates of photoreactivation of Anacystis cultures under white light illumination. Irradiation and photoreactivation were performed as described for Fig. 6. Photoreactivation was done under white light fluorescent lights. UV dosages for strains uvs-1, uvs-88, uvs-35 and wild type were 40, 100, and 120 sec, respectively.*
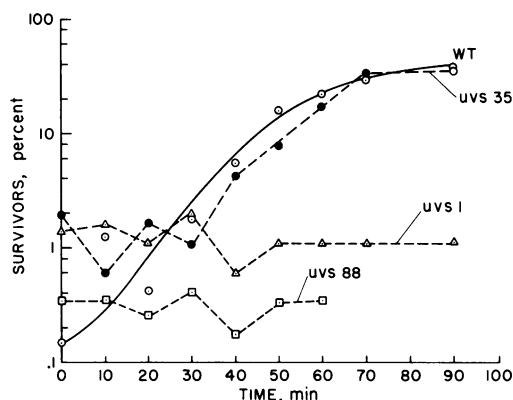

FIG. 8. *Rate of photoreactivation of chloramphenicol-pretreated cultures. Exponential cultures were treated with 1.0 µg of chloramphenicol per ml for 5 hr. Chloramphenicol was eliminated by centrifugation. UV irradiation and photoreactivation were performed as in Fig. 7. UV doses were 40, 80, 120, and 120 sec for strains uvs-1, uvs-88, uvs-35, and wild type, respectively.*

does occur in the dark as evidenced particularly by the small but significant cell increases (6%) over a period of 12 hr (2). One could argue that the postirradiation lethal effects in the dark are due to residual DNA synthesis before the damaged sites are repaired. The effect of red light could be explained by such a hypothesis since photosynthesis (and probably DNA synthesis) proceeds under red light illumination (5). It is likely that genome replication prior to repair of extensive DNA damaged sites may also be a lethal factor in *Anacystis*. However, direct experimental evidences are required to prove or disprove the notions made here.

Mutants isolated here display various degrees of UV sensitivity as evidenced by the dark-survival curves. Since strain uvs-1 was the most sensitive to UV irradiation one would expect this mutant to be a dark-repair-less mutant. However, caffeine, which inhibits dark repair in *E. coli* (12, 13), was found to depress dark-survival values in both strain uvs-1 and wild type. One can argue that the depression of the survival curve could be due to effects of caffeine pretreatment. It would be difficult to rule out this possibility since caffeine, most probably, is not permeable in the dark. The fact remains that caffeine-pretreated cultures of strain uvs-1 were comparatively more sensitive to UV as demonstrated in their response to dark survival. It is likely, therefore, that strain uvs-1 has active dark (excision) repair enzymes. Presence of dark repair in an acriflavine-treated culture of *Anacystis* was also

reported by Singh (14). If one assumes that the sensitivity of strain uvs-1 to UV irradiation is not due to dark (excision) enzymes, alternative interpretations of uvs-1 sensitivity are suggestive. First, strain uvs-1 may have lost other dark repair systems, i.e., recombination repair (10). Second, in *Anacystis*, there could be some type of protective mechanisms against UV irradiation. In this consideration, the hypothetical protective mechanism of strain uvs-1 is considered to be lost. Of the two interpretations, the second possibility is more likely. In this view, the sensitivity of strain uvs-1 could be simply explained in that it absorbs the greatest amount of UV damage as compared to other strains studied here.

Photoreactivation in vivo should be a major mode of photorepair in UV-irradiated cultures for reasons discussed in the Introduction. Photoreactivation could be detected under both black and white illumination. Note, however, that under white light illumination survival curves of mutants uvs-1, uvs-35, and uvs-88 decreased initially, and then increased thereafter. The initial decrease was reproducible for strain uvs-1, and in several instances the decreases continued over a period of 30 min before a steady increase was evident. A satisfactory explanation of this initial decrease cannot be made at this time.

The highly efficient nature of blue-green algal photoreactivation has been reported previously, although the details remain obscure. Several possibilities exist. (i) The base composition of *Anacystis* is rich in guanine plus cytosine (GC) and therefore presents fewer instances of thymine dimer formation. (ii) Since the DNA replication cycle of *Anacystis* is about 7 hr at a generation time of 8 hr at 32 C (2), photoreactivation processes are effective over an extended period. (iii) Presence of large amounts of constitutive or inducible photoreactivation enzymes per cell could account for the high capacity of photorecovery. (iv) An alternate photorecovery system, i.e., indirect photoreactivation (7), exists in *Anacystis*. (v) A combination of modes (ii), (iii), and (iv) is operative.

Statement (i) can be ruled out since the GC ratio has been reported to be 56% for *Anacystis nidulans* (4) and this value has been confirmed in this laboratory. Odd bases such as 5-methylcytosine and 6-methylaminopurine have been found in *Plectonema boryanum* in small amounts (8). The net effect of odd bases on photoreactivation remains to be tested. Similar base analysis of *Anacystis* has not been done. Statement (ii) surely should increase the

capacity of both photoreactivation and dark repair. Constitutive photoreactivation enzymes (iii) were recovered by Saito and Werbin (11), although no assessment of the number of such enzymes per cell has yet been made.

Alternative photorecovery (iv) systems may exist in *Anacystis*. First, although not shown here, preliminary studies of black light photoreactivation of *Anacystis* indicated the presence of "indirect photoreactivation" (7). However, further studies involving the use of monochromatic light at wavelengths 334 and 438 nm and analysis of dose rate and temperature independence should be made to demonstrate clearly the presence of indirect photoreactivation in *Anacystis*. Second, Van Baalen (18) suggested that the UV damage of the blue-green alga, *Agmenellum quadruplicatum*, was primarily upon the photosynthetic machinery. The recovery observed subsequently was due to the photorecovery of the photosynthetic apparatus. This photorecovery process remains to be elucidated.

Explanations of the partially photoreactivable mutants remain open. Mutants uvs-35 and uvs-88 are partially photoreactivable, although the genetic defects are different in the two. Strain uvs-88 displays partial photoreactivation under white light illumination but no photoreactivation upon pretreatment with chloramphenicol. In comparison, photoreactivation activity of strain uvs-35 was not diminished by chloramphenicol pretreatment.

In conclusion, efficient radiation repair and protective mechanisms of blue-green algae are expected since their ancestors evolved in an atmosphere of unrestricted cosmic radiations during the Precambrian era (see references 3 and 16). The high efficiencies of photorecovery explain in great part the relatively high tolerance to UV. The relatively efficient photoreactivation of *Anacystis* may be due to the presence of a high level of photoreactivation enzymes and extended DNA replication and cell division times. The extremely UV-sensitive mutant suggests the presence of a protective mechanism or other dark repair mechanisms in wild type. Biochemical analysis of the partially photoreactivable mutants should resolve whether they are leaky mutants, or whether they produce a lesser amount of photoreactivation enzymes than did the wild-type organisms, or whether two mutually exclusive photorecovery processes exist in blue-green algae.

Center.

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